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Anti-Pathogenic Activity of Coral Bacteria Againts White Plaque Disease of Coral Dipsastraea from Tengah Island, Karimunjawa

Anti-Pathogenic Activity of Coral Bacteria Againts White Plaque Disease of Coral Dipsastraea from Tengah Island, Karimunjawa

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Abstract. Coral disease is main factor of degrading coral reefs, such as White Plaque (WP) disease that cause loss of epidermal tissue of corals. The purposes of this research were to identify the bacteria associated with White Plaque Disease of coral *Dipsastraea* and to investigate coral bacteria that have antipathogenic potency against White Plaque Disease by Coral *Dipsastraea*. Sampling was carried out by purposive method in Tengah Island, Karimunjawa on March 2015. Streak method was used to isolate and purify coral bacteria, while overlay and agar diffusion method were used to test antibacterial activity. Identification of selected bacteria was conducted by biochemical and molecular methods. Polyphasic identification of bacteria associated with diseased coral White Plague of *Dipsastraea*. It is found that TFWP1, TFWP2, TFWP3 and TFWP4 were closely related to *Bacillus antracis*, *Virgibacillus olivae*, *Virgibacillus salarius* and *Bacillus mojavensis*, respectively. While antipathogen activity bacterial isolates, NM1.3, NM1.8 and NM2.3 were closely related to *Pseudoalteromonas flavipulchra*, *Pseudoalteromonas piscicida,* and *Vibrio azureus*, respectively. Phylogenetic data on microbial community composition in coral will help with the knowledge in the biological control of coral diseases.

Keywords: Coral disease, White Plaque, *Dipsastraea*, Anti-pathogen, and Molecular

1. Introduction

Coral reef ecosystems in Indonesia have highest biodiversity in the world. It is estimated that more than 3,000 species of marine life can be found in the coral reefs ecosystem [1], [2]. Eutrophication, sedimentation and over-exploitation of marine resources are the factors that cause the destruction of coral reef ecosystems. Coral disease is one of the main factors that caused degradation of coral reefs [3], [4], [5].

Coral disease was discovered in the Caribbean in the 1970s, but more recent studies conducted in the 1990s. Black Band Disease (BBD) is the first incident occurred. The discovery of the coral disease until today continues to increase rapidly and it has been reported that more than 29 species of coral syndrome have infected [6], [7], [8], [9]. Based on published literature, the coral diseases in Indonesia were found as the Black Band Disease in Karimun[10]; the coral diseases in the Wakatobi Marine National Park, Southeast Sulawesi[11], [2]; the coral disease surveillance in Spermonde Islands, South

Sulawesi[12], [13];the spread of coral disease in the Lembata, NTT[14]; the distribution and spread of the Black Band Disease (BBD) on coral *Montipora* sp. in the Seribu Islands[15]; White Band Disease on coral *Acropora humilis* and *Acropora turtosa* in Karimunjawa[16]; Pink Blotch Disease in Karimunjawa[17]; the association of bacteria in coral *Pachyseris* sp. infected BBD in Barang Lompo Island, Makassar[18]; about the coral disease in Panjang Island, Jepara[19].

Coral disease problems in Indonesia are very complex as well as the iceberg that is only slightly exposed, but each much a mystery that must be explored and researched. Further research was needed on coral diseases, especially in Balai Taman Nasional Karimunjawa, that coral diseases were found in the other side of Karimunjawa islands. The purpose of this research was to identify the bacteria associated with White Plaque Disease by coral *Dipsastraea*, determined the ability of coral symbiont bacteria activity that could potentially be antipathogen against White Plaque Disease and identifying coral symbiont bacteria biochemical and molecular potentially be antipathogen against White Plaque Disease by coral *Dipsastraea*.

2. Material and methods

2.1. Isolation Bacteria on Healthy and Disease Corals

Seven genera of corals (*Acropora, Isopora, Porites, Montipora, Stylophora, Dipsastraea, and Pocillopora*) were gathered by Scuba Gear at depths of 2 m to 5 m from Tengah Island, Karimunjawa, in March 2015 (S 05º 48,654'; E 110º 30,483'). Identification coral disease and healthy used Underwater Cards for Ascending Coral Health on Indo-Pacific Book [20]. Each specimen was placed separately into Zip-lock Plastic and placed in cool box to evade contact with air. The samples were kept each specimens on coral into Zip-lock plastic and added sea water until processing within a several hours after in the field . Surface tissue of corals were removed from skeleton with Sterilized scrapper. . Tubes used to the homogenized tissues were serially diluted, spread on half-strength ZoBell 2216E marine agar medium and incubated at Incubator (37° C) for 48 hours. On the basis of morphological features, colonies were randomly picked base on cultural characteristic (colors, configurations, margins, elevations) and purified by another plates[21].

2.2 First Screening using the overlay method

The bacterial isolates associated with healthy corals were tested for anti-pathogenic influence of the agar overlay method against selected bacterial pathogens. Each of diseased bacterial isolate was used for anti-pathogenic effect study. The 48 h, healthy corals isolates were spotted on the half-strength ZoBell 2216E marine medium agar and incubated at incubator for 2 d. All diseased isolates were cultured in ZoBell 2216E marine medium liquid and the 48 h cultures were used for the experiments. About 25 µL of the test cultures were suspended in 25 mL of ZoBell 2216E soft-medium agar and were poured immediately over the colonies of the healthy coral bacteria on the plates. The plates were incubated at incubator for 2 d. Antibacterial activity was defined by the formation of inhibition zones around the bacterial colony [22].

2.3 Rescreening of the isolates for antagonistic activity using disc-diffusion method

All bacterial strains selected from overlay test were re-screened for their activity using the agar disc diffusion method. Filter paper discs, 8 mm in diameter (Advantec, Tokyo Roshi, Ltd, Japan) were placed on the surface of plates that previously inoculated with 50 µL of each diseased isolate. Then, 25 µL of selected isolated isolates were put on each filter discs. The plates were then incubated at 37 ºC for approximately 72 h. In the end of incubation period, the zones of incubation were measured. Zone measurements were recorded as the distance from the edge of the zone to the edge of the disc [23].

2.4 Microscopic and biochemical characterizations

Selected bacterial strains that consistently active on agar diffused method were grown in ZoBell 2216E agar and underwent further microscopic and biochemical evaluations. Photomicrograph was

used to determine the morphology of the isolates. While standard gram staining, motility and biochemical characterizations based on Bergey's Manual of Determinative Bacteriology were used to determine their biochemical properties [24]. Even both microscopic and biochemical characterization were recorded, but were not presented in this paper.

2.5 DNA extraction, PCR amplification and sequencing of 16S rRNA gene fragment

DNA extraction was conducted by Chelex methods [25]. Selected colonies were inoculated in 50-100 µL ddH_2O and 1 mL of 0,5 saponin in PBS 1x (overnight in freezer). The mixture was centrifuged (12000 RPM, 10 min). Supernatant was discarded, then 100 µL ddH_2O and 50 µL of 20% Chelex 100 were added to a final solution and solution and the solution was boiled for 10 min and vortex once after 5 min. The mixture was centrifuged (12000 RPM, 10 min) and stored at -20ºC. DNA concentration were quantified and qualified by using NanoDrop 2000 spectrophotometer. DNA extracts for 16S rRNA gene sequences were amplified by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3')[26] and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3') [27]. The PCR mixture consisted KAPA mix Extract (25 µL), Primer 27F (2 µL), Primer 1492R (2 µL), DNA template (2,5 µL), and ddH_2O (18,5 µL). The PCR reaction was performed on Kyrex Thermal Cycler using cycling conditions consisting of an initial denaturation at 95ºC for 3 min followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min. The Final extension was performed at 72ºC for 7 min [28]. The PCR product was analyzed by Agarose 1% gel electrophoresis and the result showed by using UVI-Doc (UVITEC Cambridge). DNA sequencing was conducted at 1st Base, Singapura. The sequences of amplified 16 rRNA genes were deposited in the GenBank database the National Center for Biotechnology Information (NCBI). The sequence was inserted to BLAST search program to identify the sequences of any closely related organisms [29].

2.6 Phylogenic Analysis

The result of DNA sequences was preliminarily aligned with ClustalW Multiple Alignment and the phylogenetic analyses were performed by MEGA6. The phylogenetic trees were determined using the neighbor-joining method with Kimura's two-parameter. The resultant tree topology was evaluated by bootstrap analyses of the neighbor-joining method based on 1.000 resampling[30].

3. Results and discussion

3.1 Isolation and screening of anti-pathogenic coral bacteria

The total associated bacteria on each of the species from Corals was 183 strains marine bacteria associated and four bacteria from white plaque disease in *Dipsastraea* coral. Forty-four (24,04 %) had antimicrobial activity at least one to any pathogenic bacterial tested. These bacterial strains selected for overlay test were rescreened for their activity by using the agar disc diffusion method. The result that showed that 3 isolates were still consistently inhibiting the growth of pathogenic tested (Table 1).

Table 1. Mean and standard deviation (±Sd) of anti-pathogenic assay by using diffusion paper

No.	Isolate Code	Inhibition zone (mm)			
		TDWP 1	TDWP 2	TDWP 3	TDWP 4
1.	NM 1.3	8,18 ± 0,06	21,48 ± 0,17	-	6,02 ± 0,18
2.	NM 1.8	7,40 ± 0,38	22,12 ± 0,51	-	6,37 ± 0,14
3.	NM 2.3	-	-	5,10 ± 0,25	-

3.2 Molecular characterization and Phylogenetic analysis

In this regard the 16S rRNA sequencing assist resolve the exact taxonomic position of coral bacteria and provided more detailed information on their phylogenetic among their closest relatives (Table 2 and Figure 1).

Table 2. Overview of 16 rRNA gene sequences retrieved from anti-pathogenic coral bacteria

No.	Isolate	Closest species	Similarity (%)	Accession Number
1.	TDWP 1	*Bacillus anthracis*	99%	NR 041248
2.	TDWP 2	*Virgibacillus olivae*	95%	NR 043572
3.	TDWP 3	*Virgibacillus salaries*	95%	NR 041270
4.	TDWP 4	*Bacillus mojavensis*	96%	NR 118290
5.	NM 1.3	*Pseudoalteromonas flavipulchra*	95%	NR 025126
6.	NM 1.8	*Pseudoalteromonas piscicida*	96%	NR 119147
7.	NM 2.3	*Vibrio azureus*	97%	NR 041683

The result from BLAST homology of bacterial strain TDWP 1 and TDWP 4 showed that this bacterium is affiliated to the genus *Bacillus*, and bacterial strain TDWP 2 and TDWP 3 showed that this bacterium is affiliated to the genus *Virgibacillus*. Isolates TDWP 1, TDWP 2, TDWP 3, TDWP 4 come from white plaque disease on coral *Dipsastraea*.

In this study, members of the genera *Bacillus, Virgibacillus, Pseudoalteromonas* were dominant, followed by *Vibrio*. Previus studies indicated the presence of some of these genera, including *Pseudoalteromonas* sp. and *Pseudomonas* sp. Genera *Pseudoalteromonas*, as currently defined [31], comprises heterotropic, gram-negative, aerobic, rod-shaped, polarly flagellated bacteria that are common inhabitants of marine environments (seawater, *algae*, marine invertebrates) [32,33,34].

Several species of the genera *Vibrio* are ubiquitous in aquatic environments, especially the ocean, and are often isolated from various organism ranging from plankton to fish [35,36]

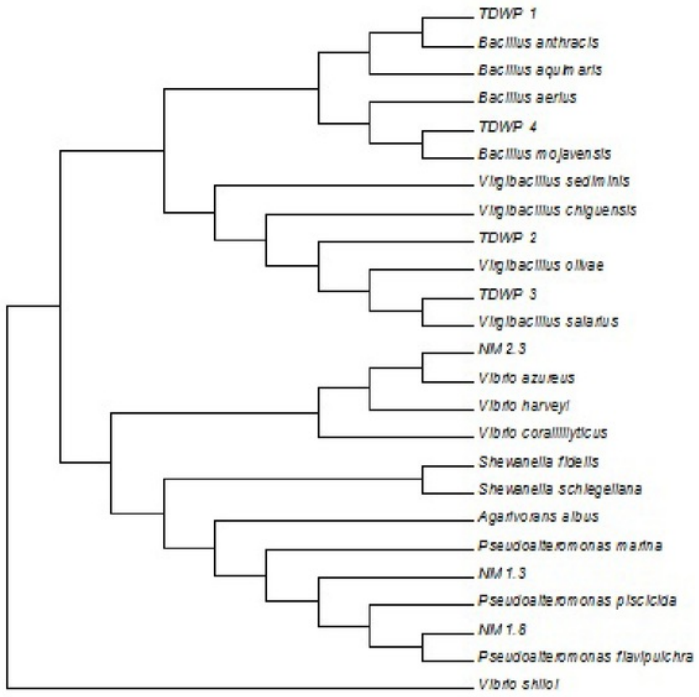

Fig 1. Neighbor-joining phylogenetic tree based on comparative 16S rRNA gene sequence analysis of seven isolates. Selected sequences *Vibrio shiloi* was used as an outer group

4. Conclusion

Three bacterial associated with corals were able to inhabit in vitro bacterial growth of bacteria isolated from white plaque disease on coral *Dipsastraea*. The molecular identification by partial 16S rRNA gene sequencing revealed that they were closely related to genera *Bacillus, Virgibacillus, Pseudoalteromonas,* and *Vibrio*.

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